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Ottawa Hull K1A 0C9

(21) (A1) 2,124,271
(22) 1994/05/25
(43) 1994/11/27

(51) INTL.CL.[5] C12N-015/21; C07K-015/26

(19)(CA) **APPLICATION FOR CANADIAN PATENT** (12)

5,091,7/89

(54) Process for Preparing and Purifying Alpha-Interferon

(72) Hauptmann, Rudolf - Austria ;
Falkner, Edgar - Austria ;
Bodo, Gerhard - Austria ;
Voss, Tilman - Germany (Federal Republic of) ;
Maurer-Fogy, Ingrid - Germany (Federal Republic of) ;

(71) Boehringer Ingelheim International GmbH - Germany (Federal Republic of) ;

(30) (DE) P 43 17 459.0 1993/05/26
(DE) P 43 29 756.0 1993/09/03

(57) 23 Claims

Abstract

The invention relates to a new process for preparing recombinant IFN-α. Expression in E. coli is carried out under the control of a phoA promotor. By linking the IFN-α gene with the STII signal sequence, the secretion of the protein into the periplasmic space and a correctly processed N-terminus are obtained. Purification of the protein is carried out by adsorption chromatography on silica gel, hydrophobic interaction chromatography, cation and anion exchange chromatography.

The invention relates to a process for preparing interferon-α (IFNα) by bacterial expression and subsequent isolation, an expression vector for this purpose and a process for purifying IFNα.

Processes for preparing IFNα by bacterial expression are known. The conventional process is based on cytoplasmic expression of the protein in Escherichia coli, in which the expressed IFNα is either present in the cell in insoluble form in so-called inclusion bodies or is found in the soluble fraction after the cell wall has been opened up (Thatcher et Panayotatos, 1986; Goeddel et al., 1980; Dworkin-Rastl et al., 1983). Cytoplasmic expression does have some disadvantages, however. The synthesised protein is not correctly folded and because reducing conditions prevail in the cytoplasm it does not contain the necessary disulphide bridges. The IFNα formed therefore has to be oxidised and re-folded during preparation. This process is inefficient and leads to unwanted by-products (wholly or partially reduced forms, oligomers produced by intermolecular disulphide bridge building, wrongly folded forms by the formation of false disulphide bridges), which are difficult to separate off. A further problem is that the N-terminal methionine with which translation begins is only partly cleaved from the IFNα synthesised intracellularly. The resulting N-Met-IFNα is almost impossible to remove from the native IFNα.

A further disadvantage of the processes currently used is the use of promotors which, in the non-induced state, are not completely switched off, which have to be induced by the addition of chemicals and which have an unsatisfactory expression rate in the induced state, such as for example the trp-promotor from Serratia marcescens.

In order to overcome some of the disadvantages mentioned and nevertheless make use of the economic E. coli system, Breitling et al. attempted (Breitling et al., 1989) to express IFNα1 and an IFNα1/2 hybrid with a vector which enabled secretion of the interferon through the cell membrane into the periplasmic space. They used a promotor, ribosome binding site (RBS) and signal sequence from a bacterial Staphylokinase gene (sak42D). 60-80% of the IFNα thus produced were secreted into the periplasmic space. However, the protein contained N-terminal amino acids which do not occur in the corresponding native IFNα, as a result of the vector construction. A serious drawback of this expression system was the fact that the strains transformed with this construct did not remain genetically stable; the expression cassette was inactivated by the spontaneous insertion of an IS1 element. The objective of providing an expression/secretion system in E. coli for preparing human IFNα has thus not been achieved in the prior art.

A known expression/secretion cassette which had been successful in the expression of human growth factor receptor in E. coli was a construct from the promotor of alkaline phosphatase (phoA) and the signal sequence of the heat-stable enterotoxin II (STII) (Fuh et al., 1990).

Another problem in the production of recombinant IFNα in E. coli is the purification of the protein from the bacterial lysate. A number of processes are known (Thatcher et Panayotatos, 1986; EP-A 203 382). In order to obtain the native folding of the protein it is preferable to use processes which do not require denaturing and precipitation steps. Such a process is described in EP-A 396 555. It consists of the steps of immunoaffinity chromatography, reversed phase chromatography (RPC), cation exchange chromatography,

concentration by ultrafiltration and gel filtration chromatography. This process, like other known processes, is based on the high selectivity of immunoaffinity chromatography in the first step. There is no known process for preparing highly purified IFNα, particularly IFNα2, which dispenses denaturing/precipitation steps and immunoaffinity chromatography. At the same time, a process of this kind is desirable for economic and technical reasons. Because of the need for monoclonal antibodies for immunoaffinity chromatography the costs are high and, at the same time, since the life of the antibody-coupled matrices is limited, a continuous supply of these antibodies is required.

The aim of the invention is to prepare a more economic and more efficient process for preparing interferon-α, particularly interferon-α2, by recombinant expression in E. coli. At the same time the problem of establishing an efficient and stable system for the expression/secretion of the protein into the periplasmic space or the culture medium had to be solved. Moreover, a process had to be developed for highly purifying the expressed protein gently, without any denaturing/precipitation steps and without the need for immunoaffinity chromatography.

This problem has been solved by means of the present invention. The establishing of a stable expression/secretion system for IFNα in E. coli was achieved by constructing a vector which contains the signal sequence (leader sequence) of heat stable enterotoxin II (STII) from E. coli, linked to the coding sequence for a mature human interferon-α, preferably interferon-α2. Preferably, expression control is effected by means of the promotor of alkaline phosphatase from E. coli (phoA). It has further proved

advantageous to integrate the ribosome binding site of the STII gene. Further surprising progress was achieved by providing a purification process for interferon-α which consists of the steps of adsorption chromatography on silica gel, hydrophobic interaction chromatography (HIC), cation exchange chromatography and anion exchange chromatography.

One aspect of the invention thus relates to a process for preparing IFNα by bacterial expression using transformed bacteria cells which contain an expression vector in which the STII signal sequence is linked to an IFNα gene, and by isolation of the expressed IFNα. According to another aspect, it relates to a bacterial expression vector for the preparation of IFNα, which contains a construct from the signal sequence of the STII gene and an IFNα gene, as well as the use of such a vector for preparing IFNα. According to a third aspect the invention relates to a process for preparing IFNα by the chromatographic steps of adsorption chromatography on silica gel, hydrophobic interaction chromatography, cation and anion exchange chromatography.

The starting point for the construction of the vector may be a plasmid which is replicable in E. coli, such as the plasmid pAT153 (Twigg et al., 1980), which is highly suitable for this purpose. A nucleotide sequence which codes for the signal peptide of the STII gene is known in the art (Picken et al., 1983; Lee et al., 1983). The person skilled in the art is in a position to prepare variants of this sequence by mutation (substitution, deletion, insertion, addition) without changing the basic properties thereof, and particularly to prepare nucleotide sequences which code for the same amino acid sequence of the signal peptide owing to the degeneration of the genetic code (Sambrook et al., 1989, especially Chapter 15). A whole series of sequences which code for

members of IFNα family are known (Mantei et al., 1980; Streuli et al., 1980; Goeddel et al., 1981); the homology of the genes which code them is more than 70%. Other variants of these sequences can be found in nature or prepared from the known sequences by methods known in the art, e.g. by mutagenesis (Sambrook et al., 1989, especially Chapter 15). The term "IFNα" according to the invention therefore includes, in addition to the known sequences, those variants whose genes are characterised by a high degree of homology with the known sequence and which code for biologically active IFNα. Particularly preferred is the sequence which codes for IFNα2c (Dworkin-Rastl et al., 1983; Bodo and Fogy, 1985). It is particularly preferred to use the phoA-promotor for controlling expression and it is also advantageous to integrate the ribosome binding site of the STII gene. The sequence of the phoA-promotor (Chang et al., 1986; Shuttleworth et al., 1981) and that of the STII ribosome binding site (Picken et al., 1983; Lee et al., 1983) are known; the person skilled in the art can also easily produce equivalent variants from these sequences. Construction of the vector, transformation of suitable E. coli strains, fermentation and extraction can be carried out using methods known per se (Sambrook et al., 1989). For example, the E. coli strain W3110 (E. coli K12 Wild type f^-, λ^-, IN (rrnD-rrnE)1) is very suitable for expression. The preliminary culture can be produced satisfactorily in LB medium and the main culture can be made, with monitoring of the supply of oxygen and nutrients, up to an OD_{546} of 250 to 280. One extraction method which has proved suitable involves suspending acid-inactivated biomass in dilute acetic acid with the aid of a homogeniser, adding polyethyleneamine, preferably in a concentration of 0.25% (w/v), adjusting the mixture to an alkaline pH, preferably pH 10, stirring the mixture and then removing the bacteria by centrifuging. Purification may be

carried out using methods known per se (Thatcher et Panayotatos, 1986; EP-A 203 382). However, a purification process with four chromatographic steps, namely adsorption chromatography on silica gel, hydrophobic interaction chromatography, cation and anion exchange chromatography, has proved particularly advantageous. Type 953W gel made by Grace proved highly suitable as the gel layer for the silica chromatography, whilst a buffer, 500-1500 mM tetramethylammoniumchloride (TMAC), preferably 800 mM TMAC, was advantageously used as eluant. For the hydrophobic interaction chromatography, phenyl sepharose proved to be satisfactory as the bed of gel. The samples were preferably applied in the presence of 20% ammonium sulphate and the column was equilibrated with a buffer containing 30% ammonium sulphate. The IFNα was eluted with a linear gradient having a final concentration of 30% ethylene glycol. Cation exchange chromatography was satisfactorily carried out using a sulphopropyl ion exchange resin. The samples were applied at a pH of 3 to 5, preferably pH 3 and the column was equilibrated to pH 5. IFNα was successfully eluted with a linear common salt gradient with the addition of 10% ethylene glycol. The gel bed used for anion exchange chromatography advantageously consisted of DEAE-sepharose and the application and elution were carried out at pH 5.5 to 6.0, preferably at pH 5.8. A linear common salt gradient with the addition of 0.1% Tween 20 proved highly suitable for elution. It is within the technical capabilities of anyone skilled in the art to replace one or more gel materials with equivalent materials based on the same separation principles, without any inventive activity, and in this way to perform an equivalent to the process according to the invention.

Surprisingly, by linking the STII signal sequence to the IFNα gene, it was possible to establish a stable

expression/secretion system, which had not been possible with the sak42D leader/IFNα combination described hereinbefore. It proved particularly successful to express this sequence under the control of the phoA promotor. The integration of the ribosome binding site of the STII gene additionally proved advantageous in this connection. Expression can reliably be controlled by monitoring the phosphate concentration in the medium (phosphate deficiency activates the phoA promotor); in the inactivated state there is no detectable basal expression. Additional chemicals do not need to be added for activation; the expression rate in the activated state is high. The synthesised protein is secreted in large amounts into the periplasmic space. The secreted protein is correctly folded, contains the authentic N-terminus and the correct disulphide bridges. The SDS gel analysis of expression in E. coli W3110 showed that 30-50% of the synthesised IFNα had been correctly processed; this corresponds to virtually all the secreted protein.

Using the extraction process described in Example 3 it was possible to extract 29.3 ± 5.9% of all the IFNα2c detectable in the biomass. This corresponded to the processing level of 30-50% which was observed. The extract from the biomass contained 4.5 ± 1.8% IFNα2c, based on total protein. Silica adsorption chromatography led to an IFNα2c pool with an average purity of 16.7 ± 4.4%. Phenyl sepharose chromatography with a yield of 93.2 ± 7.3% yielded an IFNα2c with a purity of 71.2 ± 15.5%. Sulphopropyl ion exchange chromatography produced a yield of 70.9 ± 14.8% and a purity of 97.6 ± 4.6%. The last step, namely DEAE ion exchange chromatography, resulted in 100% pure IFNα2c, in a yield of 86.9 ± 9.2%, as detailed hereinafter. The data from 6 different purifications are shown in Tables 1 (yields) and 2 (IFNα2c content). Fig. 3 shows

characteristic chromatograms of each purification step.

From 1 kg of biomass 340 ± 100 mg of purified IFNα2c were obtained. The yield of the purification process is 56.1 ± 22.2%. The total yield, based on the IFNα2c content of the biomass, is 14.4%. These data are shown in Table 3. Fig. 4 shows a typical SDS-PAGE of purified IFNα2c, eluting in the last chromatographic step. The 18 kDa band of IFNα2c is the only visible band. No contaminating bands are observed. Fig. 5A shows a typical reversed phase HPLC chromatogram. The purified IFNα2c elutes as a homogeneous peak at 24.8 minutes. When this material was eluted with a flat acetonitrile gradient (Fig. 5B), 2 contamination peaks were observed on either side of the main peak. These shoulders, which contain approximately 1.8% of the total IFNα2c content, represent forms which are oxidised at the methionine 111 (first shoulder) or acetylated at the N-terminus (second shoulder).

Table 1: Yields of various purification steps in percent of IFNα2 obtained after the purification step in question, shown for 6 different purification procedures (p1-p6) of 6 different biomasses. The last two columns contain the mean (M) and the standard deviation (sd)

	p1	p2	p3	p4	p5	p6	M	sd
Extract	37.9	24.0	34.3	30.7	29.1	20.0	29.3	5.9
Silica	62.0	95.8	88.2	99.5	74.1	81.0	83.4	12.8
Phenyl	100.0	82.2	85.9	100.0	100.0	91.0	93.2	7.3
Sulphopro	64.0	54.3	76.5	100.0	60.0	71.0	70.9	14.8
DEAE	95.0	100.0	83.5	88.2	84.0	71.0	86.9	9.2

Table 2: IFNα2 content of different purification steps. The data are shown as in Table 1, as a percentage of the IFNα2 content, based on the total protein content obtained in this purification step.

	p1	p2	p3	p4	p5	p6	M	sd
Extract	8.0	2.1	4.1	4.7	3.6	4.4	4.5	1.8
Silica	12.9	11.6	15.7	15.7	19.4	15.6	16.7	4.4
Phenyl	76.6	43.3	62.9	62.9	80.0	93.5	71.2	15.5
Sulphopro	98.5	87.3	100.0	100.0	100.0	100.0	97.6	4.6
DEAE	100.0	100.0	100.0	100.0	100.0	100.0	100.0	0.0

Table 3: Total yields of the purification process. The IFNα2c content of the biomass is shown as g of IFNα2/kg of biomass. Processing and extraction are expressed as a percentage of the total content of IFNα2. The yield of purification is shown as a percentage of IFNα2c relative to the IFNα2 content of the extract. The total yield is expressed in mg IFNα2, obtained per kg of biomass, and as a percentage of purified IFNα2c, based on the IFNα2c content of the extract.

	p1	p2	p3	p4	p5	p6	M	sd
Biomass [g/kg]	1.4	1.0	1.1	1.5	1.1	1.8	1.3	0.2
Processing [%]	50	40	40	40	20	40	38.3	8.9
Extraction [%]	37.9	24.0	34.3	30.7	29.1	20.0	29.3	4.7
Purification [%]	39.7	42.7	57.9	90	44.5	52.3	56.1	22.2
Total yield [mg]	538	206	366	480	280	258	340	120
Total yield [%]	14.3	20.3	16.6	23.9	10.9	7.4	14.4	6.9

Figures

Fig. 1: A) Gene map of pCF2. The EcoRI-BamHI fragment of pAT153 was replaced by the expression cassette for IFN-ω1.

B) Sequence of the EcoRI (destroyed)-BamHI part, which contains the phoA-promotor, STII leader + IFNω1 gene.

Fig. 2: A) Gene map of the plasmid pDH13. The SspI-PstI fragment of pAT153 was replaced by the IFNα2c expression cassette (EcoRI-PstI fragment of 2B). The β-lactamase gene is destroyed.

B) Nucleotide sequence of the EcoRI-HindIII insert of pDH13.

Fig. 3: Chromatographic purification of IFN-α2c, extracted from biomass.

A) Adsorption chromatography on silica gel. The arrow indicates elution with 800 mM tetramethylammonium chloride.

B) Hydrophobic interaction chromatography on phenylsepharose. Elution was carried out with a linear gradient of 0 to 100% of solvent B as indicated (----).

C) Sulphopropyl cation exchange chromatography. Elution was carried out with a gradient of 0 to 100% solvent B as indicated (----).

D) Anion exchange chromatography on DEAE sepharose. Elution was carried out with a gradient of 0 to 100% solvent B as indicated

(----).

The bars under the main peaks in each chromatogram indicate the pools which contain IFNα2, which were collected and used for the subsequent steps.

Fig. 4: SDS-PAGE of purified IFNα2c, stained with Coomassie blue. The figures in the left-hand margin indicate the molecular weights of the standard proteins.

Trace 1: IFNα2c standard
Trace 2: 3 µg IFNα2c
Trade 3: 6 µg IFNα2c
Trace M: molecular weight standard

Fig. 5: Characterisation of purified IFNα2c by Reversed Phase HPLC.

A) Elution of IFNα2c with a linear gradient of 20-68% solvent B in 24 minutes.

B) Elution of IFNα2c with a linear gradient of 45-53% solvent B in 30 minutes.

Examples

Example 1: Preparation of the expression vector pDH13 and cells transformed therewith

General methods

Restriction digestion of DNA with restriction endonucleases, filling reactions, phenol extraction and precipitation of DNA, agarose gel electrophoresis and elution of DNA from agarose gels, ligation of DNA molecules, transformation of bacteria and plasmid isolation from bacteria are standard procedures and were carried out as described by Sambrook et al. (1989).

Plasmids

pCF2 — pCF2 was prepared from the plasmid pAT153 (Twigg et al., 1980). It contains the promotor of alkaline phosphatase from E. coli (phoA, Chang et al., 1986; Schuttleworth et al., 1986), the coding region of the STII leader peptide (Picken et al., 1983; Lee et al., 1983) and the gene for human IFNω1 (Hauptmann et al., 1985). Fig. 1 shows the gene map of pCF2 and the sequence of the relevant section.

pER21/1 — pER 21/1 is a bacterial expression vector for IFNα2c (European Patent Specification 0 115 613)

Oligonucleotides (5'→3'):

EBI-2787: CGTCTTCAAGAATTCGAGATTATCG

EBI-2799: GGCAGATCACATGCATAGGCATTTGTAGCAATAG

EBI-2798: ATGCCTATGCATGTGATCTGCCTCAAACCCACAGC

EBI-2797: GACTTCAGAAGCTTCTGCAGTTACGATCGATCGTTATCATTCC
TTACTTCTTAAACTTTC

Preparation of the expression cassette from phoA promotor, IFNα2c sequence and STII leader sequence in a two-step PCR

pER21/1 DNA was linearised with HindIII, pCF2-DNA with PvuI. The method used hereinafter is described as SOE-PCR ("splicing by overlap extension", Ho et al., 1989).

PCR 1a (Amplification of the IFN-α2c gene): 100 ng of linearised pER21/1 DNA, 25 pmol EBI-2797 and 25 pmol EBI-2798 were subjected to thermocycles in 50 µl of buffer which contained 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.01% gelatine, 0.2 mM dATP, 0.2 mM dGTP, 0.2 mM dCTP, 0.2 mM dTTP and 1.25 units of Taq-polymerase, in a Perkin Elmer Cetus Thermocycler TC-1. After 3 minutes' incubation at 94°C, 10 cycles were performed (stage 1: 40 seconds at 94°C, stage 2: 30 seconds at 55°C, stage 3: 90 seconds at 72°C).

PCR 1b (Amplification of phoA-promotor plus STII leader sequence): 100 ng of linearised pCF2-DNA, 25 pmol EBI 2787 and 25 pmol EBI 2799 were subjected to thermocycles in the same buffer and under the same conditions as described under PCR 1a.

The resulting DNA fragments of PCR 1a (540 bp) and PCR 1b (374 bp) were gel-purified (1.2% low gelling type agarose in TBE buffer, 1 x TBE: 10.8 g Tris/l, 5.5 g boric acid/l, 0.93 g EDTA/l). The agarose fragment containing the DNA fragment in question was excised and the agarose was melted by adding 100 µl of H_2O and heating to 70°C.

PCR 2: 5 µl of each agarose/DNA solution were combined and subjected to thermocycles in 100 µl of solution containing 50 pmol of EBI-2787 and EBI-2797. The buffer was the same as described under PCR 1a. The thermocycle equipment was programmed so that a delay period of 5 minutes at 94°C was followed by 20 cycles (step 1: 40 seconds at 94°C, step 2: 30 seconds at 55°C, step 3: 5 minutes at 72°C; step 3 was extended by 5 seconds in each new cycle). After amplification the DNA was purified by phenol/chloroform extraction and ethanol precipitation. The PCR product was dissolved and cut with HindIII and EcoRI in the corresponding buffers.

Cloning of the PCR product (pDH9)

Bluescribe M13' (Stratagene, San Diego, CA, USA) was doubly cut with HindIII and EcoRI and the large fragment was gel-purified with a 1.2% agarose gel. 10 ng of Bluescribe M13' DNA and 50 ng of PCR product cut with EcoRI/Hind III were ligated in 10 µl of solution containing 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 20 mM dithiothreitol, 1 mM ATP, 50 µg/ml bovine serum albumin (BSA) and 2 units of T4-DNA-ligase (NEN), for 1 hour at 0°C and for 3 hours at ambient temperature. 8 µl of this solution were used for the transformation of competent E. coli cells of the strain JM 101 (E. coli K12, SupE, thi, Δ(lac-proAB), [F', traD36, proAB, lacIZΔM15]).

A clone was selected, the DNA was isolated and the expression cassette sequenced. The sequence corresponded precisely to the sequence expected theoretically (Fig. 2). The plasmid was designated pDH9.

Construction of the expression plasmid pDH13

pAT153 was doubly cut with SspI and PstI and the large fragment was isolated. pDH9 was cut with EcoRI and the ends were filled using the Klenow fragment of DNA polymerase 1 and the 4 dNTPs. After phenol extraction and precipitation of the linear pDH9-DNA, this DNA was cut with PstI and the fragment containing the phoA-promotor, the STII leader sequence and the IFNα2c gene was isolated from a 1% agarose gel.

10 ng of pAT153 x SspI x PstI and 30 ng of the fragment containing the expression cassette were ligated in 10 µl of solution for 5 hours at ambient temperature. 5 µl of this mixture were used to transform competent E. coli bacteria of the strain HB101. The selection of the transformed bacteria was carried out on LB agar plates (10 g tryptone/l, 5 g yeast extract/l, 5 g NaCl/l, 15 g bacto-agar/l), containing 10 µg/ml tetracycline. A gene map of pDH13 and the sequence of the relevant region is shown in Fig. 2.

Plasmid DNA from various colonies thus obtained was isolated and checked for correct composition by restriction analysis. A plasmid was selected and designated pDH13. The plasmid pDH13 was used for transforming E. coli W3110 (E. coli K12 Wild type, f^-, γ^-, IN (rrnD-rrnE)1).

Example 2: Fermentation

Preliminary culture

700 ml of autoclaved LB medium (10 g bacto-tryptone/l, 5 g bacto-yeast extract/l, 10 g NaCl/l, pH 7.0), containing 5 mg/l tetracycline, were inoculated in a 2 litre glass vessel from a stock culture so as to obtain

an OD_{546} of 0.01. The culture was incubated for 10 hours at 37°C with vigorous stirring (800 rpm) and aeration (5 fermenter volumes per minute [vvm]).

Main culture

Composition of medium

In the fermenter:

1.21	g/l	$(NH_4)_2HPO_4$
3.96	g/l	$(NH_4)_2SO_4$
6.53	g/l	K_2HPO_4
1.23	g/l	$MgSO_4 \times 7\ H_2O$
0.32	g/l	NaCl
0.25	g/l	NH_4Cl
1.0	g/l	Na_3-citrate x 2 H_2O
1.0	ml/l	Trace element concentrate
12.5	g/l	Glucose
20	mg/l	Thiamine-HCl
50	mg/l	L-tryptophan
100	mg/l	L-leucine
50	mg/l	L-methionine
5	mg/l	Tetracycline

Trace element concentrate

(Amounts per 100 ml)

3.35	g	$FeCl_3 \times 6\ H_2O$
1.09	g	$ZnSO_4 \times 7\ H_2O$
0.267	g	$CoCl_2 \times 6\ H_2O$
0.267	g	$Na_2MoO_4 \times 2\ H_2O$
0.221	g	$CuSO_4 \times 5\ H_2O$
0.333	g	H_3BO_3
1.37	g	$MnSO_4 \times H_2O$
10	ml	HCl conc.

H_2O ad 100 ml

Feeding during fermentation:

(Amounts based on volume of fermenter)

350	g/l	Glucose
3.70	g/l	$MgSO_4 \times 7\ H_2O$
175	mg/l	Thiamine-HCl
0.50	g/l	L-tryptophan
4.0	g/l	L-leucine
2.0	g/l	L-methionine

Metered addition of antifoamers during fermentation

(Based on fermenter volume)

1.0 ml/l UCON LB625

Salts $(NH_4)_2PO_4$, $(NH_4)_2SO_4$, K_2HPO_4, NaCl, NH_4Cl and Na-citrate) were sterilised in a fermenter. Trace elements, $MgSO_4$, glucose, thiamine, L-tryptophan, L-leucine, L-methionine and tetracycline were added aseptically after cooling so as to obtain a starting volume of 7 litres. 600 ml of the preliminary culture were automatically inoculated into the fermenter. The fermentation conditions were: stirring at 1000 rpm, aeration of 1 vvm, 0.3 bar ~~over~~back pressure, a temperature of 37.0 ± 0.1°C, the pH being maintained at 6.7 ± 0.1 using NH_3 and H_2SO_4. The concentration of dissolved oxygen was kept above 15% air saturation by aerating with oxygen-enriched air as necessary (at 0.3 bar backpressure ~~counterpressure overpressure~~). After the glucose initially present had been consumed, a feeding process was started up which was automatically triggered by the oxygen concentration and contained glucose, thiamine, $MgSO_4$, L-tryptophan, L-leucine and L-methionine. The

feeding rate started at 2.5 g/l/h and was increased continuously to 5.0 g/l/h within 24 hours and kept constant until the end of the fermentation process.

Fermentation was ended when a total quantity of 350 g/l of glucose had been added. At this time a typical optical density of 250 to 280 was achieved at 546 nm.

To inactive the biomass the mixture was cooled to about 10°C and at the same time the pH was adjusted to 2.0 using H_2SO_4. The biomass was separated off by centrifuging and stored frozen at -70°C.

Example 3: Extraction

Acid-inactivated biomass (about 0.5 kg) was suspended in 500 ml of 1% acetic acid using a Polytron homogeniser and the mixture was stirred for 1 hour at 0°C. Polyethyleneimine (50% stock solution, Serva, Heidelberg) was added to give a final concentration of 0.25% (w/v). The suspension was adjusted to a pH of 10.0 using 5 N NaOH and stirred for a further 2 hours at 0°C. After the pH had been adjusted to 7.5 using 5 N HCl, the bacteria were separated off by centrifuging at 17000 x g (Beckmann J2-21 centrifuge). The average extraction yield was 29.3 ± 5.9% of the total content of IFNα2c.

Example 4: Chromatographic purification

Adsorption chromatography on silica gel

The supernatant containing IFNα, after separation of the bacterial pellet in Example 3 was loaded onto a silica gel column (Grace, silica type 953W; 35 mg protein/ml column material, flow rate 25 ml/min), which had been equilibrated with 20 mM Tris-HCl, pH 7.5. The column

was washed with 30 column volumes of starting buffer, then a washing step with 20 mM Tris-HCl, 100 mM tetramethylammonium chloride (TMAC), pH 7.5, was carried out. IFNα2c could be eluted by increasing the TMAC concentration to 800 mM TMAC (Fig. 3A).

Hydrophobic interaction chromatography

The material eluted from the silica gel column was adjusted to an ammonium sulphate concentration of 20% (w/v) by the addition of solid $(NH_4)_2SO_4$ and loaded onto a phenyl sepharose column (phenyl toyopearl, 650S, Tosohaas) which had been equilibrated with 20 mM Tris-HCl, 30% ammonium sulphate. IFNα2c was eluted with a linear gradient from 100% loading conditions to 100% 20 mM Tris-HCl, 30% ethyleneglycol, pH 7.5, at a flow rate of 15 ml/min. The purity of the IFNα pool was 71 ± 15%.

Cation exchange chromatography

The eluate of the hydrophobic interaction chromatography was adjusted by extensive dialysis to 20 mM Na-succinate, pH 5.0. The final pH was adjusted to 3.0 with HCl, before the sample was loaded onto a sulphopropyl-ion exchange resin (Toyopearl TSK SP 5PW, Tosohaas), equilibrated with 20 mM Na-succinate, pH 5.0. IFNα2c was eluted from the column with a linear gradient from 100% loading conditions to 100% 20 mM Na-succinate, 500 mM NaCl, 10% ethyleneglycol, pH 5.5 (solvent B) at a flow rate of 6 ml/min. The IFNα2c eluted from this column routinely had a purity higher than 95%.

Anion exchange chromatography

The IFNα pool was dialysed against 10 mM bisTris, pH 5.8, and loaded onto a DEAE sepharose (DEAE Sepharose

FastFlow, Pharmacia) which was equilibrated with the same buffer. The elution of IFNα2c was carried out with a linear gradient on 10 mM bisTris, 500 mM NaCl, 0.1% Tween 20, pH 5.8 (solvent B), flow rate 5 ml/min.

Example 5: Analysis of the IFNα2c preparations

Reversed Phase HPLC

Intact IFNα2c was analysed at 30°C with a BakerBond -WP-C18 column [250 x 4.5 mm, particle size 5 µm). A Merck Supersphere 120-4 C-18 column [125 x 4.5 mm, particle size 4 µm) was used at 37°C to separate tryptic peptides. The samples were chromatographed using solvent A, 0.1% trifluoroacetic acid in water, and B, 0.1% trifluoroacetic acid in acetonitrile and using the gradients as described in the relevant legend to the Figure.

SDS-Polyacrylamide gel electrophoresis

IFNα2c samples were analysed on 16% SDS polyacrylamide gels under standard conditions. Samples were reduced with dithiothreitol before electrophoresis. Protein bands were visualised with Coomassie blue staining.

Quantifying IFNα2c by ELISA

The IFNα2c content of various samples obtained during purification was determined by Sandwich ELISA with the monoclonal antibodies OMG-2 and MG-7 (Adolf et al., 1990).

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Claims

1. Process for preparing interferon-α by expression in E. coli, characterised in that

a) interferon-α is expressed in cells which contain a vector in which the signal sequence of the gene for the heat stable enterotoxin II (STII) from E. coli is linked to a sequence which codes for mature human interferon-α

b) the expressed interferon-α is isolated.

2. Process according to claim 1, characterised in that the vector additionally contains a promotor for alkaline phosphatase (phoA) from E. coli.

3. Process according to claims 1 to 2, characterised in that the vector additionally contains the sequence for the ribosome binding site of the STII gene.

4. Process according to claims 1 to 3, characterised in that the isolation of the interferon comprises the steps of

a) adsorption chromatography on silica gel

b) hydrophobic interaction chromatography

c) cation exchange chromatography

d) anion exchange chromatography.

5. Process according to claim 4, characterised in that the hydrophobic interaction chromatography is carried out on a phenyl sepharose ~~column~~.

6. Process according to claim 4, characterised in that the cation exchange chromatography is carried out on a sulphopropyl ion exchanger.

7. Process according to claim 4, characterised in that the anion exchange chromatography is carried out on a DEAE-sepharose.

8. Process according to claims 1 to 7, characterised in that the interferon-α is interferon-α2.

9. Process according to claim 8, characterised in that the interferon-α2 contains the amino acid sequence

Cys Asp Leu Pro Gln Thr His Ser Leu Gly Ser Arg Arg Thr
Leu Met Leu Leu Ala Gln Met Arg Arg Ile Ser Leu Phe Ser
Cys Leu Lys Asp Arg Arg Asp Phe Gly Phe Pro Gln Glu Glu
Phe Gly Asn Gln Phe Gln Lys Ala Glu Thr Ile Pro Val Leu
His Glu Met Ile Gln Gln Ile Phe Asn Leu Phe Ser Thr Lys
Asp Ser Ser Ala Ala Trp Asp Glu Thr Leu Leu Asp Lys Phe
Tyr Thr Glu Leu Tyr Gln Gln Leu Asn Asp Leu Glu Ala Cys
Val Ile Gln Gly Val Gly Val Thr Glu Thr Pro Leu Met Lys
Glu Asp Ser Ile Leu Ala Val Arg Lys Tyr Phe Gln Arg Ile
Thr Leu Tyr Leu Lys Glu Lys Lys Tyr Ser Pro Cys Ala Trp
Glu Val Val Arg Ala Glu Ile Met Arg Ser Phe Ser Leu Ser
Thr Asn Leu Gln Glu Ser Leu Arg Ser Lys Glu

10. Process for purifying interferon-α, characterised in that it comprises the steps of

a) adsorption chromatography on silica gel

b) hydrophobic interaction chromatography

c) cation exchange chromatography

d) anion exchange chromatography.

11. Process according to claim 10, characterised in that the hydrophobic interaction chromatography is carried out on a phenyl sepharose ~~column~~.

12. Process according to claim 10, characterised in that the cation exchange chromatography is carried out on a sulphopropyl ion exchanger.

13. Process according to claim 10, characterised in that the anion exchange chromatography is carried out on a DEAE-sepharose.

14. Process according to claims 10 to 13, characterised in that the interferon-α is bacterially expressed.

15. Process according to claims 10 to 14, characterised in that the interferon-α is interferon-α2.

16. Process according to claim 15, characterised in that the interferon-α2 contains the amino acid sequence

Cys Asp Leu Pro Gln Thr His Ser Leu Gly Ser Arg Arg Thr
Leu Met Leu Leu Ala Gln Met Arg Arg Ile Ser Leu Phe Ser
Cys Leu Lys Asp Arg Arg Asp Phe Gly Phe Pro Gln Glu Glu
Phe Gly Asn Gln Phe Gln Lys Ala Glu Thr Ile Pro Val Leu
His Glu Met Ile Gln Gln Ile Phe Asn Leu Phe Ser Thr Lys
Asp Ser Ser Ala Ala Trp Asp Glu Thr Leu Leu Asp Lys Phe
Tyr Thr Glu Leu Tyr Gln Gln Leu Asn Asp Leu Glu Ala Cys
Val Ile Gln Gly Val Gly Val Thr Glu Thr Pro Leu Met Lys
Glu Asp Ser Ile Leu Ala Val Arg Lys Tyr Phe Gln Arg Ile
Thr Leu Tyr Leu Lys Glu Lys Lys Tyr Ser Pro Cys Ala Trp
Glu Val Val Arg Ala Glu Ile Met Arg Ser Phe Ser Leu Ser
Thr Asn Leu Gln Glu Ser Leu Arg Ser Lys Glu

17. Vector for expressing interferon-α in E. coli, characterised in that it contains the signal sequence of

the STII gene in conjunction with a sequence which codes for mature human interferon-α.

18. Vector according to claim 17, characterised in that it additionally contains a phoA promotor.

19. Vector according to claims 17 to 18, characterised in that it additionally contains the ribosome binding site of the STII gene.

20. Vector according to claims 17 to 19, characterised in that the interferon-α is interferon-α2.

21. Vector according to claims 17 to 19, characterised in that it contains the nucleotide sequence

TGT GAT CTG CCT CAA ACC CAC AGC CTG GGT AGC AGG AGG ACC
TTG ATG CTC CTG GCA CAG ATG AGG AGA ATC TCT CTT TTC TCC
TGC TTG AAG GAC AGA CGT GAC TTT GGA TTT CCC CAG GAG GAG
TTT GGC AAC CAG TTC CAA AAG GCT GAA ACC ATC CCT GTC CTC
CAT GAG ATG ATC CAG CAG ATC TTC AAT CTC TTC AGC ACA AAG
GAC TCA TCT GCT GCT TGG GAT GAG ACC CTC CTA GAC AAA TTC
TAC ACT GAA CTC TAC CAG CAG CTG AAT GAC CTG GAA GCC TGT
GTG ATA CAG GGG GTG GGG GTG ACA GAG ACT CCC CTG ATG AAG
GAG GAC TCC ATT CTG GCT GTG AGG AAA TAC TTC CAA AGA ATC
ACT CTC TAT CTG AAA GAG AAG AAA TAC AGC CCT TGT GCC TGG
GAG GTT GTC AGA GCA GAA ATC ATG AGA TCT TTT TCT TTG TCA
ACA AAC TTG CAA GAA AGT TTA AGA AGT AAG GAA

or a sequence which is more than 70% homologous with this sequence and codes for interferon-α.

22. Vector according to claim 21, characterised in that it contains the nucleotide sequence

GAATTCGAGATTATCGTCACTGCAATGCTTCGCAATATGGCGCAAAATGACCAACAG
CGGTTGATTGATCAGGTAGAGGGGGCGCTGTACGAGGTAAAGCCCGATGCCAGCATT
CCTGACGACGATACGGAGCTGCTGCGCGATTACGTAAAGAAGTTATTGAAGCATCCT
CGTCAGTAAAAAGTTAATCTTTTCAACAGCTGTCATAAAGTTGTCACGGCCGAGACT
TATAGTCGCTTTGTTTTTATTTTTTAATGTATTTGCTCGAGAGGTTGAGGTGATTTT
ATG AAA AAG AAT ATC GCA TTT CTT CTT GCA TCT ATG TTC GTT
TTT TCT ATT GCT ACA AAT GCC TAT GCA TGT GAT CTG CCT CAA
ACC CAC AGC CTG GGT AGC AGG AGG ACC TTG ATG CTC CTG GCA
CAG ATG AGG AGA ATC TCT CTT TTC TCC TGC TTG AAG GAC AGA
CGT GAC TTT GGA TTT CCC CAG GAG GAG TTT GGC AAC CAG TTC
CAA AAG GCT GAA ACC ATC CCT GTC CTC CAT GAG ATG ATC CAG
CAG ATC TTC AAT CTC TTC AGC ACA AAG GAC TCA TCT GCT GCT
TGG GAT GAG ACC CTC CTA GAC AAA TTC TAC ACT GAA CTC TAC
CAG CAG CTG AAT GAC CTG GAA GCC TGT GTG ATA CAG GGG GTG
GGG GTG ACA GAG ACT CCC CTG ATG AAG GAG GAC TCC ATT CTG
GCT GTG AGG AAA TAC TTC CAA AGA ATC ACT CTC TAT CTG AAA
GAG AAG AAA TAC AGC CCT TGT GCC TGG GAG GTT GTC AGA GCA
GAA ATC ATG AGA TCT TTT TCT TTG TCA ACA AAC TTG CAA GAA
AGT TTA AGA AGT AAG GAA TGATAACGATCGTAACTGCA

23. Use of the vector according to one of claims 17 to 22 for preparing interferon-α.

Fetherstonhaugh
Ottawa, Canada
Patent Agents